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Head-to-tail interactions of the coiled-coil domains regulate ClpB activity and cooperation with Hsp70 in protein disaggregation

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Abstract

The hexameric AAA+ chaperone ClpB reactivates aggregated proteins in cooperation with the Hsp70 system. Essential for disaggregation, the ClpB middle domain (MD) is a coiled-coil propeller that binds Hsp70. Although the ClpB subunit structure is known, positioning of the MD in the hexamer and its mechanism of action are unclear. We obtained electron microscopy (EM) structures of the BAP variant of ClpB that binds the protease ClpP, clearly revealing MD density on the surface of the ClpB ring. Mutant analysis and asymmetric reconstructions show that MDs adopt diverse positions in a single ClpB hexamer. Adjacent, horizontally oriented MDs form head-to-tail contacts and repress ClpB activity by preventing Hsp70 interaction. Tilting of the MD breaks this contact, allowing Hsp70 binding, and releasing the contact in adjacent subunits. Our data suggest a wavelike activation of ClpB subunits around the ring.

Introduction

Cellular machinery has evolved to prevent or reverse protein misfolding and aggregation, which are damaging to cells and tissues. Bacterial ClpB and its yeast counterpart Hsp104 are members of the Clp/Hsp100 family and function in disaggregation and refolding of protein aggregates together with Hsp70 and its co-chaperones¹⁻⁶. ClpB/Hsp104 are conserved in bacteria, fungi, plants and

mitochondria and are essential for recovery of cells from heat shock and other proteotoxic stresses^{7,8}. The oligomeric ring Hsp100 proteins thread substrates through a central channel, via binding to conserved tyrosine residues on flexible loops⁹⁻¹⁴. They belong to the AAA+ (ATPases associated with various cellular activities) superfamily of ATPases, with characteristic α and β subdomains^{15,16}. ATP binds between the two subdomains and at the subunit interface of adjacent monomers, with a catalytic Arg-finger provided by the neighbouring subunit.

While some other members of the Hsp100 family have been crystallised in their oligomeric form¹⁷⁻¹⁹, the atomic structure of ClpB is known only for the monomer (*T. thermophilus*). ClpB is composed of a N-terminal domain (ND) followed by two AAA+ domains (AAA-1 and AAA-2). An 85 Å long coil-coiled propeller, the middle domain (MD), is inserted into the small subdomain of AAA-1²⁰. It has two blades with mutationally sensitive sites at either end, termed motif 1 and motif 2. Cryo-EM reconstructions of *Tth*ClpB hexamers show a two-tiered molecule accounting for the AAA+ rings, but lack density for the ND. On the other hand, various cryo-EM studies of Hsp104 revealed the presence of an ND ring on top of the AAA+ ones. Although the overall shape and dimensions of the ClpB and Hsp104 hexamers are comparable in the various cryo-EM studies, there are substantial differences in the channel width²⁰⁻²³. Observation of narrow^{20,21} (15 to 30 Å) versus expanded^{22,23} (30 to 80 Å) cavities has led to different pseudo-atomic models of the hexamers. In one model, the AAA+ rings are compact, as in the crystal structures of other AAA+ hexamers such as p97, HslU or SV40 LTag, with the Arg-finger contacting the neighbouring subunit^{17,24,25}. In the EM maps used to build this compact model there is little or no density for the MD and it was assumed to extend radially outwards from the ring^{20,21}. In the expanded model, the AAA+ domains are more widely separated with the MD intercalated between them, preventing the canonical Arg-finger contacts^{22,23}. An attempt to localize the Hsp104 MD by genetically inserting a lysozyme resulted in a cryo-EM reconstruction of this chimera with visible density for the lysozyme, but not for the MD itself²¹. Since none of the existing cryo-EM structures allows an unambiguous localization of the MD, its position is still a matter of debate²⁶.

The MD confers unique disaggregase ability to ClpB/Hsp104^{27,28} and is required for species-specific cooperation with the DnaK-DnaJ(DnaKJ)/Hsp70-Hsp40 system²⁹⁻³¹. A direct interaction between the ClpB MD and DnaK has been shown by NMR

spectroscopy and site-specific crosslinking and involves the motif 2 tip of the MD and the ATPase domain of DnaK^{30,32}. The MD acts to repress ClpB disaggregase activity, and DnaK binding relieves this repression^{30,33}. Point mutations in the MD show that interactions between motif 2 and AAA-1 are critical for regulating ATPase and disaggregase activities^{30,33-35}. Thus, the MD plays an essential role in coupling Hsp70 interaction to ATPase regulation and substrate disaggregation in ClpB/Hsp104. It is therefore important to understand its structural role in ClpB/Hsp104 hexamers and in the context of the ClpB-DnaKJ bi-chaperone machinery.

To address the ambiguities in domain arrangement and to elucidate the working principle of the MD, we performed single particle EM studies of ClpB under conditions where its orientation could be determined more reliably than in previous studies. We used BAP (ClpB with the ClpA tripeptide for ClpP binding), a chimera engineered to bind the ClpP protease via the replacement of a C-terminal ClpB segment with the ClpP binding region of ClpA¹⁴. This construct has been extensively used to study the ClpB disaggregation mechanism by monitoring substrate proteolysis after delivery to ClpP^{9,12,14,32,36} (Figure 1A). Therefore, BAP is suitable for structural studies and has allowed us to obtain maps with visible MD densities that shed light on its regulatory mechanisms.

Results

Three-dimensional (3D) reconstruction of negatively stained BAP-ClpP shows clear density for the middle domain

Using the BAP complex facilitates orientation determination in EM reconstruction, as previously observed for ClpA-ClpP³⁷. Side views of the elongated BAP-ClpP complex are easily recognisable (Figure 1B) whereas in 2D projections of ClpB alone, which has a globular shape, it is hard to distinguish side from tilted views. By restricting the dataset to side views the angle assignment is more reliable, and these views are sufficient to generate the full 3D structure (Figure 1C). Using H/D exchange experiments, which report on the solvent accessibility and structural flexibility of amide hydrogens, we found that BAP, either alone or bound to ClpP, displays the same protection pattern as ClpB, implying the same MD conformation (Figure 1-supplement 1).

105 The BAP-ClpP complexes formed by mixing BAP and ClpP at a 1:1 molar ratio of
106 hexamer to heptamer in the presence of ATP γ S were stained with uranyl acetate.
107 Complexes containing two BAP hexamers per ClpP double-heptamer were picked for
108 single particle analysis and separated into halves for processing³⁷ (Figure 1B). Initial
109 analysis revealed the presence of four layers corresponding to the ND, AAA-1, AAA-
110 2 and ClpP rings. Class averages and eigenimage analysis indicate that the ND is
111 extremely mobile in the BAP hexamer (Figure 1-supplement 2), consistent with
112 crystallographic data on monomeric *T. thermophilus* ClpB²⁰. The ND layer was
113 therefore excluded during later stages of image alignment. Similarly, the region
114 corresponding to ClpP, very useful for the initial analysis, was not included in the
115 refinement because of its symmetry mismatch with ClpB. Using ~12,000 particles, we
116 obtained a 3D map by refining the alignment of the two AAA+ rings only, but then
117 including the whole molecule in the reconstruction, which was at ~17 Å resolution
118 (Figure 1C,D; Figure 1-supplement 3). In order to simplify the problems of alignment
119 and reconstruction, we initially imposed 6-fold symmetry, which blurs the features of
120 the heptameric ClpP ring. Similarly, the mobile ClpB ND is blurred into a solid disc.

121

122 The BAP hexamer has overall outer dimensions of ~150 × 100 Å, similar to previous
123 structures of ClpB/Hsp104^{20,22,23,38} (Figure 1C). It encloses a ~30 Å wide central
124 channel, comparable in size to that in the crystal structure of ClpC¹⁹ (Figure 1C,D).
125 In the reconstruction it is possible to identify regions accounting for all the domains,
126 such as L-shaped densities for the AAA+ domains and a rod-like density for the
127 coiled-coil MD.

128

129 To interpret the domain interactions, we fitted ClpB atomic coordinates into the EM
130 density. We determined the crystal structure of an ND truncation of *E. coli* ClpB
131 (residues 159 to 858; E432A mutation; Table 1). The subunit structure is very similar
132 to that of *T. thermophilus* ClpB (Figure 1-supplement 4). Since none of the available
133 crystal structure conformations fit in the EM map, domains were fitted as separate
134 rigid bodies connected at hinge regions (Figure 1C). For the AAA-1 ring, it was
135 possible to build a hexamer model based on the crystal structure of hexameric ClpC¹⁹
136 (PDB code 3PXG), a homologue that also displays disaggregation activity *in vitro*³⁹.
137 The resulting ClpB AAA-1 hexamer model was automatically docked into the AAA-1
138 layer as a rigid body (Figure 1D). This strategy was chosen over the fitting of a single
139 subunit followed by hexamerisation because it is expected to provide a more accurate

140 picture of the subunit interface, which is difficult to determine at the resolution of our
141 EM map. However, this approach did not work for the AAA-2 layer since the ClpB
142 AAA-2 hexamer based on the ClpC crystal structure was not compatible with the
143 density. In this case a $\sim 40^\circ$ tilt of the monomers into the plane of the ring was
144 required to obtain the optimal fit, which resembles the pseudo-atomic model of the
145 homologue ClpA AAA-2 ring³⁷. Coordinates of the *E. coli* ClpB ND (residues 1 to
146 148) were obtained from the PDB (1KHY). A single N domain was fitted manually,
147 maintaining the connection to AAA-1, and then hexamerised by applying symmetry
148 in Chimera⁵⁸.

149
150 The AAA+ rings have a central opening of ~ 30 Å and therefore are not as compact as
151 in one of the previous models^{20,21,40}, but not as expanded as in the other²². The Arg-
152 fingers are at the interface between subunits, available to catalyse hydrolysis as
153 expected from mutational studies^{28,41,42}.

154
155 The ClpB coiled-coil MDs were separately docked in the rod-shaped densities
156 surrounding the AAA-1 ring (Figure 3D), maintaining the connection to the AAA-1
157 small subdomain. The pseudo-atomic model of one ClpB subunit obtained from this
158 fitting differs from the crystal structure by rotations about the inter-domain hinge
159 points (Figure 1-supplement 4B).

160
161 In this position, the MD contacts the neighbouring AAA-1 via its motif 1, while
162 motif 2 makes intrasubunit AAA-1 interactions. This is in good agreement with recent
163 biochemical data showing protection of these two motifs upon ClpB oligomerisation
164 and formation of an intermolecular disulphide bond between E344C of AAA-1 and
165 L424C of a neighboring MD motif 1³³ (Figure 1E).

166
167 Moreover, intramolecular disulphide cross-links engineered between AAA-1 and
168 motif 2 residues in *Tth*ClpB, *Eco*ClpB and yeast Hsp104 are also compatible with the
169 fitted structure (Figure 1E; K476C/E358C³³; G175C/R484C, H362C/Q473C *E. coli*
170 numbering²⁰; G175C/S499C³⁴; Hsp104 K358C/D484C³⁵). However, this
171 arrangement is not compatible with some of the engineered disulphide bonds
172 observed in yeast Hsp104⁴³ (D427C/E475C, D427C/E471C and E320C/N467C).

173

174 To investigate possible species-specific structural differences we reconstructed the
175 functional yeast homologue HAP (Hsp104 with the ClpA tripeptide for ClpP binding)
176 in complex with ClpP. Using ~10,000 particles and analyzing the data as described
177 above, an independent map of HAP was obtained at ~21 Å resolution (Figure 2A;
178 Figure 1-supplement 3). The three-layered structure is comparable to BAP and there
179 is density accounting for all domains including the MD, which surrounds the AAA-1
180 ring. The atomic coordinates of the ND and AAA+ rings obtained from the BAP
181 analysis were fitted as rigid bodies. In order to fit the density, the MD must adopt a
182 more horizontal orientation (Figure 2A). In summary, HAP shows overall the same
183 structural organisation as its bacterial homologue ClpB.

184

185 **Cryo EM reconstructions of ClpB with and without ClpP support the negative** 186 **stain maps**

187 Since negative stain EM of ClpB (BAP) in complex with ClpP gave a clear result
188 different from all previous cryo EM maps of ClpB and Hsp104, we collected cryo EM
189 data on BAP-ClpP as well as on ClpB alone. The same strategy of using only clearly
190 identifiable side views was applied. Complexes were imaged in the presence of
191 ATP γ S and independent maps were obtained by *de novo* angular reconstitution in
192 each case (Figure 2B,C).

193 For cryo EM of the BAP-ClpP complex, we used a Trap (E279A/E678A) variant,
194 which can bind but not hydrolyse ATP due to mutations in both Walker B motifs. We
195 anticipated that the Trap construct would be more stable than the wild type, but data
196 collection was challenging because side views were not abundant. Eventually, ~4500
197 particles were collected and the same processing strategy was used as for the negative
198 stain data. This variant is more likely to trap non native substrates in its central
199 channel, and the extra density seen in this complex is likely to arise from denatured
200 protein, possibly ClpP, present in the sample (Figure 2B).

201 In the case of isolated ClpB, side views were sorted on the basis of multivariate
202 statistical analysis (MSA). Briefly, particles representing all views of ClpB were
203 picked, centered and classified by MSA. Only particles belonging to classes
204 representing side-views ($80^\circ < \beta < 100^\circ$) were extracted and used for further processing.
205 Both maps show overall dimensions and density distributions comparable to the
206 negative stain structure of BAP in complex with ClpP, confirming that the ClpB
207 hexamer structure is not significantly altered either by negative staining or by binding
208 to ClpP. The atomic model derived by fitting the negative stain BAP-ClpP map could

209 be docked as a rigid body into both cryo EM reconstructions, but the MD assumes a
210 more horizontal orientation, similar to that in HAP (Figure 2).

211

212 **ClpB activity mutants show altered MD orientations**

213 E432A and Y503D are ClpB point mutations at opposite ends of the MD coiled-coil,
214 which result in repressed (E432A) and hyperactive (Y503D) states^{30,33}. Repressed
215 ClpB-E432A is deficient in DnaK interaction and cannot be activated by its Hsp70
216 partner. Hyperactive ClpB-Y503D shows high ATPase and substrate unfolding
217 activity even in the absence of Hsp70^{30,33}. We collected negative stain EM datasets of
218 the BAP-ClpP complexes of these variants and obtained 3D reconstructions of BAP-
219 E432A and BAP-Y503D at 18 Å and 20 Å, respectively (Figure 3; Figure 1-
220 supplement 3). Starting models were independently (Figure 3-supplement 1)
221 generated by angular reconstitution and refined with 6-fold symmetry. Both mutants
222 assemble into three layers similar to the wild type and show high variability in the ND
223 ring. Atomic coordinates of the AAA+ rings can be fitted as described for the wild
224 type, using the ClpC hexamer as starting point. Some rearrangement was necessary to
225 fit the ND into density (Figure 3). The most notable difference between the two maps
226 is a ~30° difference in orientation of the motif 1 blade of the MD and the loss of the
227 motif 2 density in the hyperactive state (Figure 4A,B). Another difference observed
228 upon alignment of the AAA-1 ring in the repressed, wild-type and hyperactive maps
229 is a ~15° rotation of the wild-type AAA-2 ring relative to the mutants.

230

231 In the BAP-E432A repressed mutant the MD is clearly visible around the AAA-1
232 ring. It has a horizontal orientation so that motif 2 forms a contact with motif 1 of the
233 neighbouring MD (Figure 3B; Figure 4B). This packing of the MD is supported by
234 biochemical data showing overprotection of motif 2 in this mutant³³. Moreover, a
235 very similar intersubunit motif 1-motif 2 contact is seen in all the ClpB crystal
236 structures, in which ClpB monomers are arranged in a spiral assembly (*Tth*ClpB,
237 1QVR²⁰; *Eco*ClpB current study) (Figure 4-supplement 1).

238

239 In the Y503D hyperactive form there is density for motif 1 in a more tilted
240 orientation, but there is no density visible for motif 2, suggesting that this region
241 becomes either disordered or highly mobile (Figure 3C,D). This finding is in
242 accordance with H/D exchange data showing deprotection of this region in the BAP-
243 Y503D mutant³³. We conclude that, in the Y503D hyperactive form, the motif 1 arm

244 of the MD is tilted downwards so that it can no longer contact motif 2 of the adjacent
245 MD, and binds to a lower region of the neighbouring AAA-1 domain. Motif 2 is
246 mobile and solvent exposed (Figure 3C,D; Figure 4A,B).

247

248 Alignment of AAA-1 domains of wild type, repressed and hyperactive forms of
249 ClpB/BAP confirms that the MD rotates $\sim 30^\circ$ from a tilted orientation in the
250 hyperactive state, to a horizontal one in the repressed state, with the wild type
251 occupying an intermediate orientation (Figure 4B). In this movement motif 1 switches
252 from being protected against AAA-1 to engaging motif 2 in trans. These different
253 orientations of the MD have opposite consequences for DnaK binding. The horizontal
254 MD position, with motif 1 contacting motif 2 of the neighbouring subunit, is
255 incompatible with DnaK binding at the tip of motif 2^{30,32}. In contrast, tilting of the
256 MD exposes motif 2 for DnaK interaction.

257

258 To confirm the close proximity of motif 1 and motif 2 of neighbouring MDs we
259 performed fluorescence resonance energy transfer (FRET) experiments using Q427W
260 (motif 1) as FRET donor and IAEDANS labelled Q502C (motif 2) as FRET acceptor.
261 The FRET pair (Förster radius of 22 Å) has a distance of 19.1 Å in the wild type
262 model and was introduced into the tryptophan-free ClpB-W462Y/W543L variant. In
263 addition we coupled the FRET pair to E432A and Y503D mutations, to monitor the
264 consequences of repressed and hyperactive states on FRET efficiency. The distance
265 between the FRET partners is either reduced (11.3 Å) or increased (35.9 Å) in models
266 of the repressed and hyperactive variants, respectively (Figure 4B). All ClpB variants
267 were IAEDANS labeled with similar efficiencies (70-80%; Figure 4-supplement 2).
268 High IAEDANS fluorescence and thus FRET efficiency was observed for wild type
269 ClpB and E432A upon ClpB oligomerization. Increase in acceptor fluorescence in
270 general correlated with reduced tryptophan emission except for ClpB wild type upon
271 oligomerization. In contrast, IAEDANS fluorescence remained low under all
272 conditions tested when the FRET pair was linked to Y503D (Figure 4C).

273

274 Furthermore, to test for direct interaction between motif 1 and motif 2, we introduced
275 cysteine residues into motif 1 (K431C) and motif 2 (S499C) and analyzed whether
276 intermolecular crosslinks form under oxidizing conditions. Low Cu-Phenanthroline
277 concentrations (25 μ M) yielded a ladder of crosslink products from ClpB dimers to
278 hexamers. Although non-specific dimer formation was observed in single cysteine

279 variants, higher oligomers were only found in double cysteine variants (Figure 4D).
280 Introducing the activating (Y503D) mutation to ClpB-K431C/S499C decreased but
281 did not abolish crosslink formation, suggesting rapid fluctuation of MDs between
282 different conformations even in the hyperactive state (Figure 4D). These findings
283 support the interaction of MD motifs 1 and 2 from adjacent subunits as observed in
284 the EM reconstructions and strengthening or loosening of the contact in repressed and
285 hyperactive states, respectively.

286

287 **Motif 1 is essential for keeping ClpB in the repressed state**

288 Mutating MD motif 1 can impair Hsp70 interaction and consequently protein
289 disaggregation^{30,33}. On the other hand a ClpB motif 1 deletion variant retains
290 substantial disaggregation activity^{30,44}, providing no clarification of its role in protein
291 disaggregation. Our EM structural data indicate that motif 1 acts as a crucial
292 component of ClpB regulation by stabilizing MDs in a horizontal position through
293 interaction with an adjacent motif 2. Indeed, ClpB-ΔM1 (ΔE410-Y455) shows
294 hyperstimulation of ATPase activity in the presence of the substrate casein compared
295 to wild-type ClpB, resembling the deregulated ATPase activity of hyperactive ClpB
296 variants lacking motif 2 or the entire MD³³ (Figure 5A). The basal ATPase activity of
297 ClpB-ΔM1 was reduced in comparison to wild type due to partial oligomerization
298 defects, in agreement with earlier reports^{33,44}. ClpB-ΔM1 oligomerization defects
299 were more pronounced than in ClpB-ΔM1/M2, suggesting that motif 2 in absence of
300 motif 1 might impede hexamer formation.

301 Next we tested whether hyperstimulation of ATPase activity is linked to high
302 unfolding power. We employed BAP variants of the respective deletion constructs
303 and tested for unfolding and degradation of casein-YFP in presence of ClpP. BAP-
304 ΔM1 unfolds the YFP moiety of casein-YFP, an activity only observed for
305 hyperactive BAP variants but not wild type BAP³³ (Figure 5B). Loss of ClpB
306 regulation in ClpB-ΔM1 was also linked to severe toxicity upon expression in *E. coli*
307 Δ*clpB* mutant cells (Figure 5C). However, higher expression levels of ClpB-ΔM1
308 than of ClpB-Y503D are required to observe the same degree of toxicity probably
309 because deletion of motif 1 results in oligomerisation defects³³ (Figure 5C). In
310 conclusion ClpB-ΔM1 exhibits the three major characteristics of hyperactive ClpB
311 variants (high ATPase and unfolding activities linked to cellular toxicity),
312 demonstrating the crucial regulatory role for motif 1 in interacting with an adjacent
313 motif 2 to ensure tight activity control of ClpB.

314 **Asymmetric reconstructions show variable orientations of the MD around the**
315 **ring**

316 As mentioned above, we imposed 6-fold symmetry as a first approximation, to
317 simplify the alignment and reconstruction problem. Nevertheless, crystal structures of
318 the AAA+ protein ClpX show that the homo-hexameric assembly can be markedly
319 asymmetric^{18,45,46}. We therefore reanalysed our negative stain EM data without
320 imposing symmetry, in order to study the conformational variability within the
321 hexamer.

322 Using ~15,000, ~10,000 and ~9000 particles for wild-type BAP, BAP-E432A and
323 BAP-Y503D we obtained asymmetric maps at 21 Å, 21 Å and 25 Å resolution,
324 respectively (Figure 6A; Figure 1-supplement 3; Figure 6-supplement 1). Although
325 the numbers of particles were similar to those used for 6-fold analysis, the resolution
326 is only slightly worse and the maps are comparable in quality to the symmetrised
327 ones, consistent with the structure being asymmetric. The structure of the hyperactive
328 mutant is less defined and has lower resolution than the other two, particularly for the
329 MDs, probably owing to their higher mobility. Therefore, only wild-type BAP and
330 BAP-E432A were used for further analysis.

331
332 The asymmetric structures show similar density distributions in the AAA+ rings, even
333 though not all six subunits in a ring can be aligned simultaneously owing to 5°-15°
334 variations in rotational orientation. This rotational variability may explain the
335 apparent ~15° rotation of the AAA-2 wild-type ring relative to the mutants observed
336 in the symmetrised reconstructions. As expected from the eigenimage analysis, the
337 ND ring is not very well defined and the density does not account for all six NDs.
338 Therefore, we did not attempt any atomic structure fitting into this region. Docking of
339 the atomic structures of AAA-1, AAA-2 and MD was performed as follows.

340
341 Structures of AAA+ proteins crystallised in their hexameric assemblies show that the
342 large and small subdomains of the AAA fold assume a range of conformations that
343 can be clustered into open or closed forms^{46,47} (Figure 6-supplement 2). The closed
344 conformation is ATP-binding competent and there are intermediate forms that might
345 also represent weak binding states. Using a gallery of available AAA+ crystal
346 structures we modelled open, closed and intermediate conformations of ClpB AAA-1
347 and AAA-2. We also created AAA+ dimers of adjacent ClpB subunits based on ClpX
348 pseudo-hexameric crystal structures^{18,46} (Figure 6-supplement 2) that were fitted as

349 rigid bodies into the asymmetric reconstructions. Crystallographic dimers are likely to
350 provide more realistic models of subunit interfaces than can be deduced by fitting
351 individual subunits into low-resolution maps.

352

353 The AAA-2 ring density could be almost entirely interpreted using this approach and
354 the fit suggests that 3 to 5 subunits are sufficiently closed to bind ATP (Fig. 6-
355 supplement 2). We analyzed nucleotide (ADP) binding to wild type ClpB by
356 isothermal calorimetry (ITC) revealing a binding stoichiometry of 7.5 ± 0.1 ADP
357 per hexamer (Figure 6-supplement 3). ITC experiments using ClpB-K212A, which is
358 deficient in nucleotide binding in AAA-1, allowed us to determine a binding
359 stoichiometry of 3.7 ± 0.3 ADP in AAA-2 of the mutant hexamer (Figure 6-
360 supplement 3). The same ADP binding stoichiometry was also found for the repressed
361 and hyperactive variants (Figure 6-supplement 3). The deduced stoichiometries are in
362 good agreement with the distinct AAA-2 conformations observed in the asymmetric
363 EM reconstructions. Similar calculations have been reported for the AAA+ proteins
364 MCM, ClpX and HslU^{48,49}. The AAA-1 ring is more asymmetric than AAA-2 and it
365 is not easily interpretable by fitting crystallographic dimer models. There is sufficient
366 density to guide rigid body fitting of all AAA-1 domains, but this fitting does not
367 allow deductions of nucleotide occupancy (Figure 6-supplement 2).

368

369 Asymmetric reconstructions of both wild-type BAP and repressed mutant display
370 clear densities accounting for the MDs that lie outside the AAA-1 ring and assume
371 different orientations similar to those observed in the symmetrised maps of wild type
372 and mutants. In the wild-type asymmetric reconstruction, the MD orientation ranges
373 from horizontal as in the repressed state to highly tilted, similar to the hyperactive
374 state, passing through the intermediate wild type-like state (Figure 6B). In one
375 subunit, motif 1 contacts AAA-2 of the same subunit, suggesting a route of allosteric
376 communication between AAA-1 and AAA-2. Additionally, this contact is compatible
377 with recently reported engineered disulphide bonds between motif 1 residues and
378 AAA-2 in Hsp104 and ClpB⁴³. The tilted MDs are clustered together, consistent with
379 the release of motif 1-motif 2 contacts, freeing adjacent subunits.

380

381 In the asymmetric reconstruction of the repressed state, five MDs are found in the
382 horizontal orientation, followed by a sixth for which there is no clear density
383 (Figure 6C). In the wild type two MDs can make the motif 1 to motif 2 contact, while

384 in the repressed state up to five MDs are compatible with this contact (Figure 6B,C).
385 Conversely, in the wild type at least two MDs exist in a clearly activated state but in
386 the repressed state none of the MDs are visible in the tilted, hyperactive conformation.
387 As a consequence, the MD conformations in BAP-E432A do not support Hsp70
388 binding and ATPase activation, in agreement with previous findings^{30,33}. However,
389 ClpB wild type hexamers harbour two MDs that favour Hsp70 recruitment,
390 potentially priming the ClpB ring for further activation.

391

392 **Discussion**

393 The BAP construct of ClpB complexed with ClpP, combined with a conservative
394 approach of using only clearly identifiable side views and basing the analysis mainly
395 on negative stain images (with independent confirmation from cryo EM) enabled us
396 to unambiguously locate all subunit domains in the oligomer, including the coiled-coil
397 MD propeller. Although the quoted resolutions for some previous structures of ClpB
398 and Hsp104 were better, the globular shape, flexibility and asymmetry of these
399 hexamers reduce the reliability of orientation assignment. Moreover, previous
400 published structures of ClpB/Hsp104 were obtained by cryo EM only, which provides
401 a lower signal-to-noise ratio (SNR) than negative stain data.

402

403 Rigid body fitting of *E. coli* ClpB atomic coordinates (PDB code 4CIU) into our new
404 maps reveals that the MD is not projecting outwards from the hexamer^{20,21,40} nor is
405 intercalated between subunits^{22,23}, but is instead lying on the surface of the ClpB
406 hexameric ring with a variable degree of tilt (Figure 4A; Figure 6B,C). A similar
407 position of the MD is seen for Hsp104, underlining the conserved activity and
408 mechanism of these disaggregases. This new MD arrangement is in excellent
409 agreement with recent, extensive biochemical analysis of the MD³³. It was proposed
410 that the MD works as a molecular toggle switching from a repressed state in which
411 both motif 1 and 2 ends of the propeller are protected, to an active state where motif 2
412 is deprotected, exposing the binding site for DnaK/Hsp70^{30,32,33}. Our EM
413 reconstructions of repressed, hyperactive and wild type ClpB reveal a lever-like
414 movement of the MD that switches from the repressed state with head-to-tail motif 1-
415 motif 2 binding between adjacent subunits, to a mobile, activated state with motif 2
416 free and available for binding to DnaK (Figure 7A). The wild type conformation is
417 intermediate between these states and thus poised for switching.

418

419 Our data thus explain the critical role of motif 1, which regulates the accessibility of
420 motif 2 and consequently ClpB activity. Confirming its important regulatory role,
421 deletion of motif 1 results in hyperactive ATPase and unfolding activity of ClpB-ΔM1
422 as well as cell toxicity (Figure 5), representing key characteristics of the hyperactive
423 state³³. Moreover, our maps suggest that motif 1, through its contacts with either the
424 adjacent motif 2 or AAA-1, plays a key role in direct communication between
425 neighbouring subunits, which must therefore act in a coordinated manner.

426

427 It has become clear that AAA+ proteins are highly dynamic molecular motors
428 unlikely to exist in a homogeneous structural state. Therefore we generated
429 asymmetric reconstructions of ClpB. Although the resolution of the asymmetric
430 structures is not sufficient to support a detailed mechanistic model, these
431 reconstructions provide the first visualization of the MD conformational flexibility
432 that was inferred from biochemical analysis^{20,33,34}. The asymmetric structures show
433 that the MD orientation varies around the ring occupying the repressed, wild type-like
434 and hyperactive positions described by the symmetrised averages. The variable tilts of
435 MDs observed around the ring suggest that 2 to 4 adjacent subunits are available for
436 DnaK binding in the wild-type versus only 1 in the repressed mutant (Figure 6B,C).
437 This is consistent with the estimated stoichiometry of 2-5 molecules of DnaK per
438 ClpB hexamer required for activation^{30,43}. It also suggests that at least 4 subunits
439 must have detached MDs to allow activity, perhaps through movements of the AAA+
440 domains, in agreement with the number of ClpX subunits that hydrolyze ATP in a
441 coordinated manner to unfold GFP in single molecule experiments⁵⁰. Moreover, we
442 calculated an ADP binding stoichiometry of 4 for both wild type and mutants
443 (Figure 6-supplement 3), which indicates that although ATP hydrolysis is strongly
444 affected, detachment of the MD does not change the nucleotide binding.

445

446 The cryo EM reconstruction of wild-type ClpB suggests that the various MD
447 conformations exist only transiently, poised between hyperactive and repressed states,
448 with the balance shifted slightly towards repressive motif 1-motif 2 contacts
449 (Figure 2). DnaK binding to an accessible motif 2 stabilizes the MD in a tilted
450 conformation, thereby in turn breaking the repressive contacts with MDs in
451 neighbouring subunits. Thus, an initial encounter of DnaK will facilitate DnaK
452 binding in the neighbouring MDs. In this model, breakage or formation of motif 1-
453 motif 2 contacts provides a mechanistic basis for signalling DnaK binding or

454 dissociation in a wavelike manner around the ClpB ring. (Figure 7B). The model
455 predicts spatial proximity of multiple DnaK molecules, which is the case for
456 aggregated but not soluble DnaK substrates, directing ClpB activity specifically to
457 protein aggregates. Moreover, the activation of ClpB by DnaK binding, combined
458 with movements of the highly mobile N domain, might act to deliver the substrate to
459 the channel entrance, where it would be engaged for threading, unfolding and
460 consequent extraction from the aggregate.

461

462

463 **Material and methods**

464 **Strains, plasmids and proteins**

465 *E. coli* strains used were derivatives of MC4100. Mutant derivatives of ClpB/BAP
466 were generated by PCR mutagenesis and standard cloning techniques in pDS56 and
467 were verified by sequencing. Wild type and mutant ClpB were purified using Ni-IDA
468 (Macherey-Nagel) and size exclusion chromatography (Superdex S200, Amersham)
469 following standard protocols. Purifications of DnaK, DnaJ, GrpE, ClpP, Luciferase
470 and Casein-YFP were performed as described previously³³. Pyruvate kinase and α -
471 casein were purchased from Sigma. Protein concentrations were determined with the
472 Bio-Rad Bradford assay.

473

474 **Electron microscopy of negatively stained and vitrified specimens**

475 BAP(HAP)-ClpP complexes were formed in 20 mM Tris-HCl, pH 7.5, 20 mM KCl,
476 15 mM MgCl₂, 1 mM DTT and 2 mM ATP γ S. Proteins were applied to glow-
477 discharged carbon coated grids (EM sciences), previously coated with 5 kDa poly-
478 lysine (Sigma-Aldrich) to positively charge the surface. Samples were stained with
479 2% uranyl acetate.

480 For cryo-EM imaging, BAPtrap-ClpP was applied to holey carbon grids coated with a
481 thin carbon film and pretreated with poly-lysine while ClpB specimens were loaded
482 onto lacey carbon grids. Cryo-EM specimens were vitrified in a Vitrobot (FEI).
483 Images were recorded on a 4k Gatan CCD camera at a magnification of 50,000 \times for
484 negatively stained specimens (pixel size 2.2 Å; underfocus range: 0.5-1.2 μ m) and of
485 80,000 \times for cryo specimens (pixel size 1.4 Å; underfocus range: 1.5 μ m-4 μ m). All
486 data were collected on a Tecnai F20 FEG operated at 200 kV under low dose
487 conditions.

488

489 **Single particle processing**

490 The contrast transfer function (CTF) for each CCD frame was determined with
491 CTFFIND3⁵¹ and corrected by phase flipping using Bshow1.6⁵². Side views of BAP-
492 ClpP 2:2 complexes were manually picked using Boxer⁵³ and extracted into 256×256
493 boxes. The boxed particles were band-pass filtered between 300 and 10 Å for the
494 negative stain dataset and between 300 and 5 Å for the cryo dataset. They were then
495 normalized to the same mean and standard deviation. Particles were initially aligned
496 to the total sum of 10-20 vertically oriented particles using SPIDER⁵⁴. Individual 1:1
497 BAP-ClpP complexes were extracted with circular masks and classified by MSA in
498 IMAGIC-5⁵⁵ to remove images that did not represent BAP-ClpP, yielding 17470
499 particles of wild type BAP-ClpP, 12588 of BAP-E432A-ClpP, 9436 of BAP-Y503D-
500 ClpP and 12568 of HAP-ClpP. The BAPtrap-ClpP cryo dataset included 4592
501 particles. At this stage, particles were high-pass filtered to 160 Å and initial class
502 averages of 20-30 images each were obtained by MSA. All alignments of 1:1 BAP-
503 ClpP complexes were done limiting the in-plane rotation to +/- 20°. Upon further
504 classification, 5-10 good classes were used to generate a starting model by angular
505 reconstitution⁵⁶. Alternative starting models were also created by applying 6 fold
506 symmetry to single classes and then using the resultant 3D map, generally composed
507 of three discs corresponding to the three layers of the molecule, to generate an anchor
508 set for Euler angle assignment. A low-resolution density map was independently
509 created for each dataset by angular reconstitution with 6-fold symmetry. Particle
510 orientations were refined in multiple cycles of AP SHC alignment in SPIDER, MSA
511 and angular reconstitution in IMAGIC and the resulting 3D reconstruction, filtered to
512 30 Å, was used as an initial model for projection matching in SPIDER. By applying a
513 rectangular mask, only the AAA+ layers were refined. After 8-10 cycles of projection
514 matching, using progressively smaller angular sampling steps (4° to 1°) and filtering
515 the 3D to the estimated resolution at each cycle, final structures were generated of the
516 whole complex and their resolution was estimated by Fourier shell correlation with a
517 0.5 correlation cutoff. Based on cross correlation coefficient, around 70-80% of each
518 dataset was included in the final reconstruction.

519 The differences between the mutant structures were tested by refining with
520 interchanged starting models. In both cases, the original mutant structure was
521 recovered despite the use of the other map as a starting model for projection matching
522 (Figure 3-supplement 1).

For cryo EM ClpB specimen, the views were randomly oriented and the initial strategy was to extract clearly identifiable side views by MSA and classification. 7606 side views were used to generate a starting model by angular reconstitution, which was refined to 29 Å resolution (Figure 1-supplement 3) by projection matching. For the reconstructions without imposed symmetry, particle orientations were determined using either the 6-fold symmetrized map filtered to 50 Å or a sphere obtained from the average of all particles as a starting model. Subsequently, particle orientations were refined with ~10 cycles of projection matching without imposed symmetry. The particle orientations were well distributed around the BAP-ClpP central axis (Figure 6-supplement 1). The resolution was estimated by Fourier shell correlation (FSC) at 0.5 (Figure 1-supplement 3).

534

535 **Atomic Structure Fitting**

Docking was done using the crystal structures of *E. coli* ClpB-E432A (current study, PDB code: 4CIU) and *E. coli* ClpB ND (PDB code 1KHY). A homology model of Hsp104, obtained using Phyre2⁵⁷ was used for fitting into the HAP reconstruction. A hexameric ClpB/Hsp104 AAA-1 ring was modelled on the ClpC hexamer crystal structure (PDB code 3PXG) and was automatically fitted as a rigid body into the symmetrised maps using the UCSF Chimera package⁵⁸. The N-terminal, the middle and the AAA-2 domains were first fitted manually and then local fitting was optimized in Chimera, followed by symmetrisation. In the asymmetric reconstructions, dimers of adjacent ClpB subunits were modelled on crystal structures of ClpX hexamers and fitted as rigid bodies in Chimera.

546

547 **Biochemical assays**

Steady-state ATP hydrolysis rates were determined in buffer A (50 mM Tris pH 7.5, 5 mM MgCl₂, 20 mM KCl, 2 mM DTT) as described³⁴. ClpB disaggregation activities were determined by following the refolding of aggregated firefly Luciferase according to published protocols³⁴. Chaperones were used at the following concentrations: 1 μM ClpB (wild type or derivatives), 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE. Oligomerisation of ClpB variants was tested as described previously^{28,34}. Site-specific labelling of ClpB using 1,5-IAEDANS, 5-[(2-iodoacetyl)amino]ethylamino)naphthalene-1-sulfonic acid (Invitrogen) was performed according to the manufacturers' instructions. The intrinsic tryptophan fluorescence of ClpB variant harbouring a single tryptophan (ClpB*-Q427CW, 1 μM

each) was measured on a Perkin-Elmer LS50B spectrofluorimeter at 25°C in low salt buffer A (50 mM Tris pH 7.5, 5 mM MgCl₂, 20 mM KCl, 2 mM DTT). Tryptophan and IAEDANS emission spectra of labeled ClpB variants were recorded in high and low salt buffer A (supplemented with 400 mM KCl or 20 mM KCl, respectively) in the absence or presence of 2 mM nucleotide between 300 and 550 nm at a fixed excitation wavelength of 290 nm. The Förster radius of the Trp-IAEDANS FRET pair was calculated as 22 Å⁵⁹.

For formation of disulfide bridges ClpB cysteine variants were first dialyzed to remove DTT. Cysteine oxidation was achieved by adding 25 µM Cu-Phenanthroline to 4 µM ClpB and incubating the mixture for 1 min at room temperature. Oxidation and disulfide bond formation was stopped by addition of 50 mM iodoacetamide and SDS-sample buffer containing 5 mM EDTA. Crosslink products were analyzed by a non-reducing SDS gradient gel (3-8%).

Casein-YFP unfolding and degradation assays were carried out using 6 µM BAP (wild type or variants), 9 µM ClpP and 0.5 µM Casein-YFP. Degradation of Casein-YFP was determined by monitoring YFP fluorescence at 535 nm (excitation wavelength 515 nm) at a Perkin-Elmer LS50B spectrofluorimeter.

Spot tests

E. coli cells harbouring plasmid-encoded *clpB* alleles were grown in the absence of IPTG overnight at 30°C. Serial dilutions were prepared, spotted on LB-plates containing different IPTG concentrations and incubated for 24 h at 37°C.

Isothermal titration calorimetry

ClpB wild type and variants were extensively dialyzed against low salt buffer A (50 mM Tris (pH 7.5), 25 mM KCl, 20 mM MgCl₂, 5% glycerol). Isothermal titration calorimetry (ITC) was performed using an ITC calorimeter (iTC200Microcalorimeter, MicroCal). Consecutive injections of nucleotide into a 300 µl cell containing ClpB were performed after sample equilibration at 30°C. Integration and fitting of ITC data were performed using ORIGIN software (GE). ClpB and ADP concentrations were determined by UV absorbance at 280 nm.

Hydrogen-exchange experiments

HX-MS experiments were performed similar to those described earlier³³. Briefly, ClpB (100 pmol), BAP (100 pmol) or BAP-ClpP complex (100 pmol and 200 pmol

respectively) were incubated for 3 min at 30°C in low salt buffer A (50 mM Tris, pH 7.5, 25 mM KCl, 20 mM MgCl₂, 2 mM DTT) in presence of ATP or ATP γ S and diluted 20-fold into D₂O-based MDH buffer to initiate amide proton-deuteron exchange. The exchange reaction was stopped after 1 min by the addition of 1 volume of ice-cold quench buffer (0.4 M K-phosphate buffer, pH 2.2). Quenched samples were immediately injected into the HPLC setup, with (peptide analysis) or without (full length protein analysis) online peptic digest, and analysed on an electrospray ionization quadrupole time-of-flight mass spectrometer (QSTAR Pulsar, Applied Biosystems) as described in Rist et al, 2003⁶⁰. Analysis of deuteron incorporation into peptide was performed by using AnalystQS software (Applied Biosystems/MDS SCIEX).

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Accession Numbers

The EM maps have been deposited in the 3D-EM database (www.emdatabank.org) with accession codes EMD-2555 (BAP-E432A C6), EMD-2556 (BAP-E432A C1), EMD-2557 (BAP wild type C6), and EMD-2558 (BAP wild type C1), EMD-2559 (BAP-Y503D C6), EMD-2560 (BAP-Y503D C1), EMD-2561 (HAP), EMD-2562 (BAPtrap cryo) and EMD-2563 (ClpB wild type cryo). The corresponding FSC curves have also been deposited.

625 **References**

- 626 1. Doyle, S.M. & Wickner, S. Hsp104 and ClpB: protein disaggregating machines.
627 *Trends in Biochemical Sciences* **34**, 40-8 (2009). doi: 10.1016/j.tibs.2008.09.010.
- 628 2. Glover, J.R. & Lindquist, S. Hsp104, Hsp70, and Hsp40: a novel chaperone system
629 that rescues previously aggregated proteins. *Cell* **94**, 73-82 (1998).
630 doi: 10.1016/S0092-8674(00)81223-4
- 631 3. Goloubinoff, P., Mogk, A., Zvi, A.P., Tomoyasu, T. & Bukau, B. Sequential
632 mechanism of solubilization and refolding of stable protein aggregates by a
633 bichaperone network. *Proceedings of the National Academy of Sciences of the United*
634 *States of America* **96**, 13732-7 (1999). doi: 10.1073/pnas.96.24.13732
- 635 4. Haslberger, T., Bukau, B. & Mogk, A. Towards a unifying mechanism for
636 ClpB/Hsp104-mediated protein disaggregation and prion propagation. *Biochemistry*
637 *and cell biology = Biochimie et biologie cellulaire* **88**, 63-75 (2010).
638 doi: 10.1139/o09-118.
- 639 5. Motohashi, K., Watanabe, Y., Yohda, M. & Yoshida, M. Heat-inactivated proteins
640 are rescued by the DnaK.J-GrpE set and ClpB chaperones. *Proceedings of the*
641 *National Academy of Sciences of the United States of America* **96**, 7184-9 (1999).
642 doi: 10.1073/pnas.96.13.7184
- 643 6. Zolkiewski, M. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein
644 aggregation. A novel multi-chaperone system from Escherichia coli. *The Journal of*
645 *biological chemistry* **274**, 28083-6 (1999). doi: 10.1074/jbc.274.40.28083
- 646 7. Mogk, A. et al. Identification of thermolabile Escherichia coli proteins: prevention
647 and reversion of aggregation by DnaK and ClpB. *The EMBO journal* **18**, 6934-49
648 (1999). doi: 10.1093/emboj/18.24.6934
- 649 8. Parsell, D.A., Kowal, A.S., Singer, M.A. & Lindquist, S. Protein disaggregation
650 mediated by heat-shock protein Hsp104. *Nature* **372**, 475-8 (1994).
651 doi:10.1038/372475a0
- 652 9. Haslberger, T. et al. Protein disaggregation by the AAA+ chaperone ClpB involves
653 partial threading of looped polypeptide segments. *Nature structural & molecular*
654 *biology* **15**, 641-50 (2008). doi: 10.1038/nsmb.1425.
- 655 10. Kim, Y.I., Burton, R.E., Burton, B.M., Sauer, R.T. & Baker, T.A. Dynamics of
656 substrate denaturation and translocation by the ClpXP degradation machine.
657 *Molecular Cell* **5**, 639-48 (2000). doi: 10.1016/S1097-2765(00)80243-9
- 658 11. Lum, R., Tkach, J.M., Vierling, E. & Glover, J.R. Evidence for an
659 unfolding/threading mechanism for protein disaggregation by *Saccharomyces*
660 *cerevisiae* Hsp104. *The Journal of biological chemistry* **279**, 29139-46 (2004). ,
661 doi: 10.1074/jbc.M403777200

- 662 12. Tessarz, P., Mogk, A. & Bukau, B. Substrate threading through the central pore of the
663 Hsp104 chaperone as a common mechanism for protein disaggregation and prion
664 propagation. *Molecular Microbiology* **68**, 87-97 (2008). doi: 10.1111/j.1365-
665 2958.2008.06135.x.
- 666 13. Weber-Ban, E.U., Reid, B.G., Miranker, A.D. & Horwich, A.L. Global unfolding of a
667 substrate protein by the Hsp100 chaperone ClpA. *Nature* **401**, 90-3 (1999).
668 doi:10.1038/43481
- 669 14. Weibezahn, J. et al. Thermotolerance requires refolding of aggregated proteins by
670 substrate translocation through the central pore of ClpB. *Cell* **119**, 653-65 (2004).
671 doi: 10.1016/j.cell.2004.11.027
- 672 15. Erzberger, J.P. & Berger, J.M. Evolutionary relationships and structural mechanisms
673 of AAA+ proteins. *Annual Review of Biophysics and Biomolecular Structure* **35**, 93-
674 114 (2006). doi: 10.1146/annurev.biophys.35.040405.101933
- 675 16. Ogura, T. & Wilkinson, A.J. AAA+ superfamily ATPases: common structure--
676 diverse function. *Genes to cells : devoted to molecular & cellular mechanisms* **6**,
677 575-97 (2001). doi: 10.1046/j.1365-2443.2001.00447.x
- 678 17. Bochtler, M. et al. The structures of HsIU and the ATP-dependent protease HsIU-
679 HsIV. *Nature* **403**, 800-5 (2000). doi:10.1038/35001629
- 680 18. Glynn, S.E., Martin, A., Nager, A.R., Baker, T.A. & Sauer, R.T. Structures of
681 asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-
682 unfolding machine. *Cell* **139**, 744-56 (2009). doi: 10.1016/j.cell.2009.09.034
- 683 19. Wang, F. et al. Structure and mechanism of the hexameric MecA-ClpC molecular
684 machine. *Nature* **471**, 331-5 (2011). doi: 10.1038/nature09780
- 685 20. Lee, S. et al. The structure of ClpB: a molecular chaperone that rescues proteins from
686 an aggregated state. *Cell* **115**, 229-40 (2003). doi: 10.1016/S0092-8674(03)00807-9
- 687 21. Lee, S., Sielaff, B., Lee, J. & Tsai, F.T. CryoEM structure of Hsp104 and its
688 mechanistic implication for protein disaggregation. *Proceedings of the National*
689 *Academy of Sciences of the United States of America* **107**, 8135-40 (2010).
690 doi: 10.1073/pnas.1003572107.
- 691 22. Wendler, P. et al. Atypical AAA+ subunit packing creates an expanded cavity for
692 disaggregation by the protein-remodeling factor Hsp104. *Cell* **131**, 1366-77 (2007).
693 doi: 10.1016/j.cell.2007.10.047
- 694 23. Wendler, P. et al. Motor mechanism for protein threading through Hsp104. *Molecular*
695 *Cell* **34**, 81-92 (2009). doi: 10.1016/j.molcel.2009.02.026.
- 696 24. Gai, D., Zhao, R., Li, D., Finkielstein, C.V. & Chen, X.S. Mechanisms of
697 conformational change for a replicative hexameric helicase of SV40 large tumor
698 antigen. *Cell* **119**, 47-60 (2004). doi: 10.1016/j.cell.2004.09.017

- 699 25. Huyton, T. et al. The crystal structure of murine p97/VCP at 3.6Å. *Journal of*
700 *Structural Biology* **144**, 337-48 (2003). doi: 10.1016/j.jsb.2003.10.007
- 701 26. Desantis, M.E. & Shorter, J. The elusive middle domain of Hsp104 and ClpB:
702 location and function. *Biochimica et Biophysica Acta* **1823**, 29-39 (2012).
703 doi: 10.1016/j.bbamcr.2011.07.014.
- 704 27. Kedzierska, S., Akoev, V., Barnett, M.E. & Zolkiewski, M. Structure and function of
705 the middle domain of ClpB from Escherichia coli. *Biochemistry* **42**, 14242-8 (2003).
706 doi: 10.1021/bi035573d
- 707 28. Mogk, A. et al. Roles of individual domains and conserved motifs of the AAA+
708 chaperone ClpB in oligomerization, ATP hydrolysis, and chaperone activity. *The*
709 *Journal of biological chemistry* **278**, 17615-24 (2003). doi: 10.1074/jbc.M209686200
- 710 29. Miot, M. et al. Species-specific collaboration of heat shock proteins (Hsp) 70 and 100
711 in thermotolerance and protein disaggregation. *Proceedings of the National Academy*
712 *of Sciences of the United States of America* **108**, 6915-20 (2011).
713 doi: 10.1073/pnas.1102828108.
- 714 30. Seyffert, F. et al. Hsp70 proteins bind Hsp100 regulatory M domains to activate
715 AAA+ disaggregase at aggregate surfaces. *Nature structural & molecular biology* **19**,
716 1347-55 (2012). doi: 10.1038/nsmb.2442.
- 717 31. Sielaff, B. & Tsai, F.T. The M-domain controls Hsp104 protein remodeling activity
718 in an Hsp70/Hsp40-dependent manner. *Journal of Molecular Biology* **402**, 30-7
719 (2010). doi: 10.1016/j.jmb.2010.07.030.
- 720 32. Rosenzweig, R., Moradi, S., Zarrine-Afsar, A., Glover, J.R. & Kay, L.E. Unraveling
721 the mechanism of protein disaggregation through a ClpB-DnaK interaction. *Science*
722 **339**, 1080-3 (2013). doi: 10.1126/science.1233066.
- 723 33. Oguchi, Y. et al. A tightly regulated molecular toggle controls AAA+ disaggregase.
724 *Nature structural & molecular biology* **19**, 1338-46 (2012). doi: 10.1038/nsmb.2441.
- 725 34. Haslberger, T. et al. M domains couple the ClpB threading motor with the DnaK
726 chaperone activity. *Molecular Cell* **25**, 247-60 (2007).
727 doi: 10.1016/j.molcel.2006.11.008
- 728 35. Lipinska, N. et al. Disruption of ionic interactions between the nucleotide binding
729 domain 1 (NBD1) and middle (M) domain in Hsp100 disaggregase unleashes toxic
730 hyperactivity and partial independence from Hsp70. *The Journal of biological*
731 *chemistry* **288**, 2857-69 (2013). doi: 10.1074/jbc.M112.387589.
- 732 36. Mizuno, S., Nakazaki, Y., Yoshida, M. & Watanabe, Y.H. Orientation of the amino-
733 terminal domain of ClpB affects the disaggregation of the protein. *The FEBS journal*
734 **279**, 1474-84 (2012). doi: 10.1111/j.1742-4658.2012.08540.x.
- 735 37. Effantin, G., Ishikawa, T., De Donatis, G.M., Maurizi, M.R. & Steven, A.C. Local
736 and global mobility in the ClpA AAA+ chaperone detected by cryo-electron

737 microscopy: functional connotations. *Structure* **18**, 553-62 (2010).
738 doi: 10.1016/j.str.2010.02.016.

739 38. Parsell, D.A., Kowal, A.S. & Lindquist, S. Saccharomyces cerevisiae Hsp104 protein.
740 Purification and characterization of ATP-induced structural changes. *The Journal of*
741 *biological chemistry* **269**, 4480-7 (1994).

742 39. Schlothauer, T., Mogk, A., Dougan, D.A., Bukau, B. & Turgay, K. MecA, an
743 adaptor protein necessary for ClpC chaperone activity. *Proceedings of the National*
744 *Academy of Sciences of the United States of America* **100**, 2306-11 (2003).
745 doi: 10.1073/pnas.0535717100

746 40. Lee, S., Choi, J.M. & Tsai, F.T. Visualizing the ATPase cycle in a protein
747 disaggregating machine: structural basis for substrate binding by ClpB. *Molecular*
748 *Cell* **25**, 261-71 (2007). doi: 10.1016/j.molcel.2007.01.002

749 41. Biter, A.B., Lee, J., Sung, N., Tsai, F.T. & Lee, S. Functional analysis of conserved
750 cis- and trans-elements in the Hsp104 protein disaggregating machine. *Journal of*
751 *Structural Biology* **179**, 172-80 (2012). doi: 10.1016/j.jsb.2012.05.007

752 42. Yamasaki, T., Nakazaki, Y., Yoshida, M. & Watanabe, Y.H. Roles of conserved
753 arginines in ATP-binding domains of AAA+ chaperone ClpB from *Thermus*
754 *thermophilus*. *The FEBS journal* **278**, 2395-403 (2011). doi: 10.1111/j.1742-
755 4658.2011.08167.x.

756 43. Desantis, M.E. et al. Conserved Distal Loop Residues in the Hsp104 and ClpB
757 Middle Domain Contact Nucleotide-binding Domain 2 and Enable Hsp70-dependent
758 Protein Disaggregation. *The Journal of biological chemistry* **289**, 848-67 (2014).
759 doi: 10.1074/jbc.M113.520759.

760 44. del Castillo, U. et al. A quantitative analysis of the effect of nucleotides and the M
761 domain on the association equilibrium of ClpB. *Biochemistry* **50**, 1991-2003 (2011).
762 doi: 10.1021/bi101670s

763 45. Kon, T. et al. The 2.8 Å crystal structure of the dynein motor domain. *Nature* **484**,
764 345-50 (2012). doi: 10.1038/nature10955.

765 46. Stinson, B.M. et al. Nucleotide binding and conformational switching in the
766 hexameric ring of a AAA+ machine. *Cell* **153**, 628-39 (2013).
767 doi: 10.1016/j.cell.2013.03.029

768 47. Cho, C. & Vale, R.D. The mechanism of dynein motility: insight from crystal
769 structures of the motor domain. *Biochimica et Biophysica Acta* **1823**, 182-91 (2012).
770 doi: 10.1016/j.bbamcr.2011.10.009.

771 48. Moreau, M.J., McGeoch, A.T., Lowe, A.R., Itzhaki, L.S. & Bell, S.D. ATPase site
772 architecture and helicase mechanism of an archaeal MCM. *Molecular Cell* **28**, 304-14
773 (2007). doi: 10.1016/j.molcel.2007.08.013

- 774 49. Yakamavich, J.A., Baker, T.A. & Sauer, R.T. Asymmetric nucleotide transactions of
775 the HslUV protease. *Journal of Molecular Biology* **380**, 946-57 (2008).
776 doi: 10.1016/j.jmb.2008.05.070.
- 777 50. Sen, M. et al. The ClpXP protease unfolds substrates using a constant rate of pulling
778 but different gears. *Cell* **155**, 636-46 (2013). doi: 10.1016/j.cell.2013.09.022
- 779 51. Mindell, J.A. & Grigorieff, N. Accurate determination of local defocus and specimen
780 tilt in electron microscopy. *Journal of Structural Biology* **142**, 334-347 (2003).
781 doi: 10.1016/S1047-8477(03)00069-8
- 782 52. Heymann, J.B. & Belnap, D.M. Bsoft: Image processing and molecular modeling for
783 electron microscopy. *Journal of Structural Biology* **157**, 3-18 (2007).
784 doi: 10.1016/j.jsb.2006.06.006
- 785 53. Ludtke, S.J., Baldwin, P.R. & Chiu, W. EMAN: Semiautomated software for high-
786 resolution single-particle reconstructions. *Journal of Structural Biology* **128**, 82-97
787 (1999). doi: 10.1006/jsbi.1999.4174
- 788 54. Frank, J. et al. SPIDER and WEB: Processing and visualization of images in 3D
789 electron microscopy and related fields. *Journal of Structural Biology* **116**, 190-199
790 (1996). doi: 10.1006/jsbi.1996.0030
- 791 55. van Heel, M., Harauz, G., Orlova, E.V., Schmidt, R. & Schatz, M. A new generation
792 of the IMAGIC image processing system. *Journal of Structural Biology* **116**, 17-24
793 (1996). doi: 10.1006/jsbi.1996.0004
- 794 56. van Heel, M. et al. Single-particle electron cryo-microscopy: towards atomic
795 resolution. *Quarterly Reviews of Biophysics* **33**, 307-69 (2000).
- 796 57. Kelley, L.A. & Sternberg, M.J. Protein structure prediction on the Web: a case study
797 using the Phyre server. *Nature protocols* **4**, 363-71 (2009). doi: 10.1038/nprot.2009.2.
- 798 58. Pettersen, E.F. et al. UCSF Chimera--a visualization system for exploratory research
799 and analysis. *Journal of computational chemistry* **25**, 1605-12 (2004).
800 doi: 10.1002/jcc.20084
- 801 59. Jeganathan, S., von Bergen, M., Brutlach, H., Steinhoff, H.J. & Mandelkow, E.
802 Global hairpin folding of tau in solution. *Biochemistry* **45**, 2283-93 (2006).
803 doi: 10.1021/bi0521543
- 804 60. Rist, W., Jørgensen, T.J.D., Roepstorff, P., Bukau, B., & Mayer, M.P. Mapping
805 temperature-induced conformational changes in the *Escherichia coli* heat shock
806 transcription factor σ^{32} by amide hydrogen exchange. *Journal of Biological*
807 *Chemistry*
808

809 **Figure captions**

810

811 **Figure 1.** 3D structure of the symmetrised BAP-ClpP complex. (A) Schematic of the
 812 complex showing the threading of a polypeptide. ClpB domains are in yellow, N-terminus;
 813 red/orange, AAA-1 domain; green, MD and blue/cyan, AAA-2 domain. ClpP is in grey. (B)
 814 Negative stain EM image (left panel), class averages of 2:2 BAP-ClpP (upper right panel) and
 815 cut out 1:1 BAP-ClpP particles (bottom right panel). Scale bars are 150 Å. (C) BAP-ClpP
 816 structure with a fitted ClpB monomer (left) and central slice with fitted atomic coordinates
 817 (right). (D) AAA-1 and AAA-2 layers. (E) Two adjacent AAA-1 domains from the
 818 hexameric fit viewed in the plane of the ring. One monomer is in colour and the other in grey.
 819 Residues involved in engineered disulphide bonds are shown as spheres. Residues involved in
 820 intermolecular cross-links are labeled. The separation between cross-linking Cα pairs ranges
 821 from 8 to 14 Å.

822

823 **Figure 2.** Independently determined maps and fitted hexameric models of HAP-ClpP,
 824 BAPtrap-ClpP and ClpB. (A) Negative stain EM map of HAP-ClpP. From left to right:
 825 surface side-view, AAA-1 layer and AAA-2 layer. The central channel enclosed by the AAA-
 826 2 ring is filled with density. (B) Cryo EM map of BAP-ClpP formed with the BAP variant
 827 that traps the substrate inside. (C) Cryo EM map of wild type ClpB alone.

828

829 **Figure 3.** Structures of repressed and hyperactive BAP-ClpP mutants. (A) Side view (left)
 830 and 35 Å section (right) of the BAP-E432A map with the ClpB hexamer fit. (B) Fitting of the
 831 BAP-E432A AAA+ rings. (C) Side view (left) and 35 Å section of the fitted BAP-Y503D
 832 structure. MD is only partially in density and is more tilted than in the wild type and the BAP-
 833 E432A repressed mutant. The ND is smeared out as in all BAP forms due to disorder, but has
 834 a more vertical orientation in the hyperactive state. (D) Fitting of the BAP-Y503D AAA+
 835 rings. The motif 2 region protrudes from the density.

836

837 **Figure 4.** Analysis of MD movements. (A) Repressed (red), wild-type (yellow) and
 838 hyperactive (green) forms of BAP-ClpP with a fitted subunit. (B) Distances (d) are shown
 839 between Cα atoms of Q427 (motif 1) and Q502 (motif 2) of neighbouring subunits based on
 840 the symmetrised EM reconstructions of wild-type ClpB and MD mutants. (C) Fluorescence
 841 energy transfer between motif 1 and motif 2 of adjacent ClpB subunits. Emission spectra of
 842 ClpB-Q427W-Q502C-IAEDANS in the monomeric (high salt), oligomeric (low salt) and
 843 ATP state (2 mM ATP) are shown. Effects of MD mutations on FRET efficiency were
 844 determined. The ratio of acceptor to donor fluorescence (derived from the areas under the
 845 curves) was calculated as readout for FRET efficiency. (D) Intermolecular disulfide
 846 crosslinking between MD motif 1 and motif 2 of adjacent subunits. Reduced and oxidized

847 ClpB-K431C-S499C and repressed (E432A) and hyperactive (Y503D) variants were
848 analyzed by non-reducing SDS-PAGE. Positions of monomers (1) and crosslinked dimers (2),
849 trimers (3), tetramers (4), pentamers (5) and hexamers (6) are indicated. A protein standard is
850 given. CoPhe at 25 μ M was used as oxidizing agent.

851

852 **Figure 5** Deletion of MD motif 1 causes ClpB activation. (A) Basal and substrate-stimulated
853 ATPase activities of ClpB wild type and indicated MD deletions were determined in the
854 absence and presence of casein. Relative ATPase activations by casein were calculated
855 (inset). (B) Unfolding of Casein-YFP by BAP wild type and indicated MD deletions in the
856 presence of ClpP was monitored by YFP fluorescence. Initial YFP fluorescence was set to
857 100%. Δ M1: Δ E410-Y455, Δ M2: Δ S456-E520, Δ M1/M2: Δ E410-E520. (C) *E. coli* Δ clpB
858 cells expressing the indicated plasmid-encoded *clpB* alleles under control of an IPTG-
859 regulatable promoter were grown overnight at 30°C. Various dilutions (10^{-1} – 10^{-7}) were
860 spotted on LB plates containing the indicated IPTG concentration and incubated at 37°C for
861 24 h.

862

863 **Figure 6.** Variations in MD orientations around the ring. (A) Asymmetric reconstructions of
864 repressed BAP-E432A, wild type BAP-ClpP and hyperactive BAP-Y503. (B) Side views of
865 the BAP-ClpP wild type structure rotated successively 60°. Red MDs are oriented
866 horizontally and make motif 1 to motif 2 contact as in the repressed state. Yellow MDs have
867 slightly tilted orientations similar to the wild type. Green MDs are tilted as in the hyperactive
868 state. One of the MDs contacts AAA-2 of the same subunit (last right panel). (C) Equivalent
869 views of the BAP E432A-ClpP repressed mutant structure. Color code for the MD
870 orientations is the same as above.

871

872 **Figure 7.** Interaction between the ClpB hexamer and DnaK. (A) Binding of DnaK is only
873 possible when the MD is tilted (green). DnaK cannot bind when two adjacent MDs occupy a
874 more horizontal position (red) as it would clash with motif 1 of the neighbouring ClpB
875 subunit. (B) Cartoon of an opened-out ClpB ring with two DnaK molecules bound. The MDs
876 involved in DnaK binding are shown in green, and those that cannot bind to DnaK are shown
877 in red. Release of the ends of the MD favours activation of adjacent subunits, and the active
878 cluster can move around the ring in a wave-like manner. The model of DnaK binding to the
879 MD is based on the NMR study in ref. 32.

880

881 **Figure 1 supplement 1** Complex formation between BAP and ClpP does not change MD
882 protection pattern in HX experiments. (A) BAP and ClpP form a stable complex during HX
883 experiments causing stabilization of BAP. Protein samples preincubated with ATP γ S (BAP,
884 BAP-ClpP) were diluted 20-fold into D₂O buffer and incubated for 60 s before quenching the

885 proton-deuteron exchange reaction. The mass of full length BAP after deuteron incorporation
886 was determined. Complex formation with ClpP protects 30 amide protons in BAP from
887 exchange. **(B)** ClpB, BAP and BAP-ClpP were incubated with ATP γ S and HX values of
888 peptic peptides were determined after 60 s. Peptide 746-754 (*, KKIFRPEFI) includes the
889 ClpP-binding loop of ClpA and is only present in BAP. Relative (%) deuterium incorporation
890 levels are given for each peptide.

891

892 **Figure 1 supplement 2** The BAP N-terminus is highly mobile. **(A)** Class averages of BAP-
893 ClpP. The arrows indicate the N-terminal (yellow), AAA-1 (red), AAA-2 (blue) and ClpP
894 (black) tiers. **(B)** Eigen images showing that high variance is localized in the N-terminal ring.
895 Scale bars are 50 Å.

896

897 **Figure 1 supplement 3** Fourier Shell Correlation plots to estimate EM map resolution. For
898 each sample, FSC curves of C6 and C1 reconstructions are plotted together. The C1 curves
899 show lower resolution in each pair. Example class averages are shown in the left column with
900 corresponding re-projections on the right. FSC plots were deposited in the 3D-EM database
901 EM (www.emdatabank.org).

902

903 **Figure 1 supplement 4** Crystal structure of *E. coli* ClpB and EM based model.
904 **(A)** Superimposed chains of *E. coli* ClpB and *T. thermophilus* crystal structures. For clarity,
905 the N-terminus is not shown. **(B)** Comparison between the ClpB coordinates fitted into the
906 EM maps (in colours) and the ClpB crystal structure (grey). In the EM model the MD is more
907 tilted than in the crystal structures. AAA-2 is rotated by $\sim 25^\circ$, and the N-terminus is rotated
908 by 45° in the plane of the ring.

909

910 **Figure 3 supplement 1** Test of EM map refinements after interchange of starting models.

911

912 **Figure 4 supplement 1** Contacts between adjacent MDs in the EM model compared to those
913 found in the ClpB crystal structures.

914

915 **Figure 4 supplement 2** Tryptophan emission spectra of unlabeled ClpB-Q427W-Q502C and
916 of corresponding MD mutants in the monomeric (HS, high salt), oligomeric (LS, low salt)
917 and ATP-loaded state (LS + 2 mM ATP) are given. High salt (400 mM KCl) prevents ClpB
918 oligomerisation.

919

920 **Figure 6 supplement 1** Plots showing the angular distribution of single particles (red dots)
921 around the Euler sphere for each asymmetric reconstruction. Angle search was done with 2°

922 sampling. The equatorial band reflects the Euler angle distribution around the BAP-ClpP
923 central axis.

924

925 **Figure 6 supplement 2** Open and closed conformations of AAA+ domains can be fitted into
926 the asymmetric ClpB map. (A) Superimposition of an open (blue) and a closed (grey)
927 nucleotide pocket (ClpX 3HWS). ADP is depicted in green spheres. (B) *EcoClpB* AAA-2
928 dimers built with open, intermediate and closed conformations based on ClpX
929 crystallographic dimers. (C) AAA-1 and AAA-2 layers of asymmetric wild-type ClpB
930 reconstruction plus indication of nucleotide occupancy.

931

932 **Figure 6 supplement 3** Asymmetric nucleotide binding to ClpB. (A-D) Instrumental
933 responses of successive injections of ADP (8.39 mM, 1.92 mM, 5.82 mM and 4.05 mM ADP
934 for ClpB-WT, ClpB-K212A, ClpB-E432A, ClpB-Y503D, respectively) into a solution of
935 ClpB-WT (678 μ M), ClpB-K212A (239 μ M), ClpB-E432A (467 μ M) and ClpB-Y503D (396
936 μ M). The molar ratio of ADP to ClpB hexamer is indicated. Raw isothermal titration
937 calorimetry data are shown. Integrated data after base-line-correction and fitting to the
938 respective binding isotherms to a single-site binding model are given (lower panel). The
939 number of ADP binding sites was calculated.

940

941

942

943

944

945 **Table 1: X-ray data collection and refinement statistics**

946

947 Protein ClpB E279A/E432A/E678A (SeMet) + ATP

948

949 Wavelength (Å) 0.9794

950 Space group P6₅

951 Unit cell (Å, °) 127.34, 127.34, 119.86, 90, 90, 120

952 Molecules 1

953 Resolution (Å) 81.15-3.50 (3.69-3.50)

954 Reflections measured 202965

955 Unique reflections 14024

956 Rmerge 8.8 (53.2)

957 Rpim 3.0 (14.9)

958 I/σI 16.8 (5.4)

959 Completeness (%) 99.9 (100)

960 Redundancy 14.5 (14.8)

961 Rwork/Rfree (%) 22.5/24.5

962 Protein residues 657 (5257 atoms)

963 ADP 2

964 Rmsd bond lengths (Å) 0.004

965 Rmsd bond angles (°) 0.94

966 Average B-Factor Protein 99.6

967 Average B-Factor ADP 87.7

968

969 Ramachandran plot statistics

970 Favored (%) 95.5

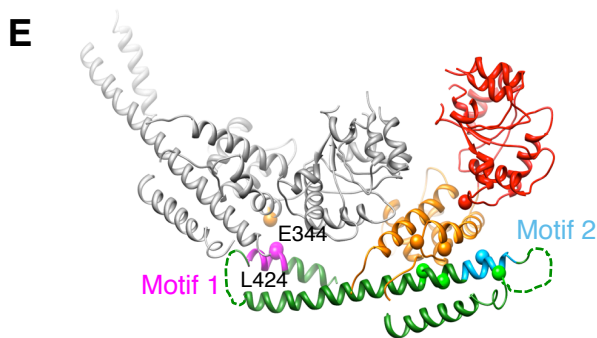
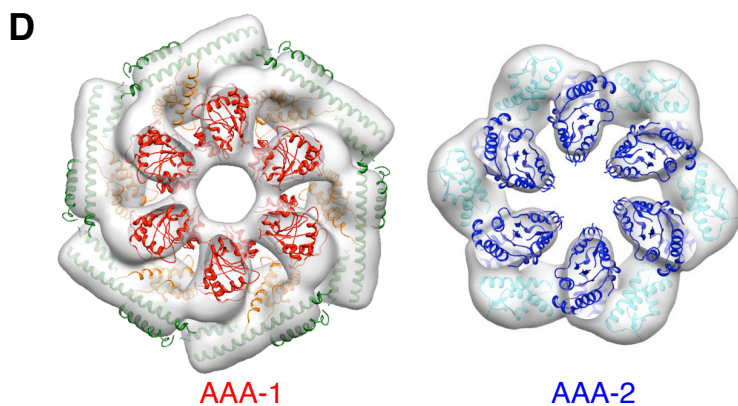
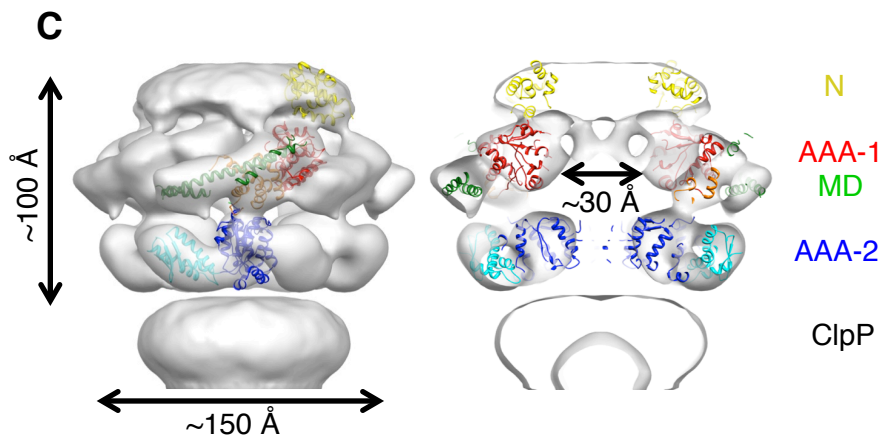
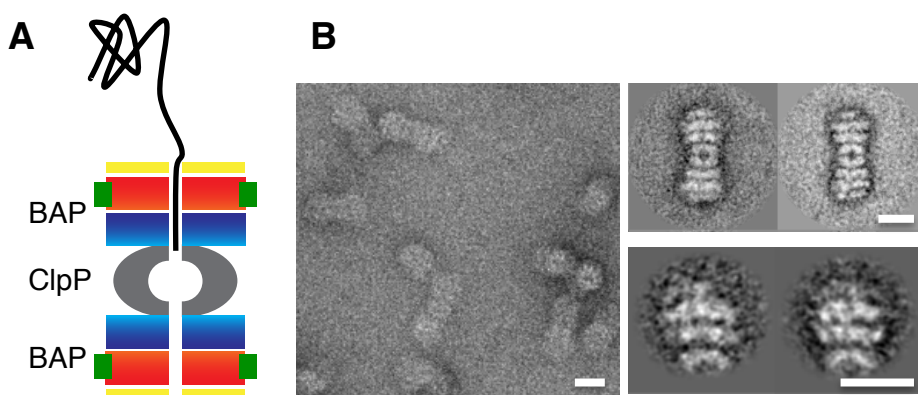
971 Allowed (%) 4.1

972 Generous (%) 0.4

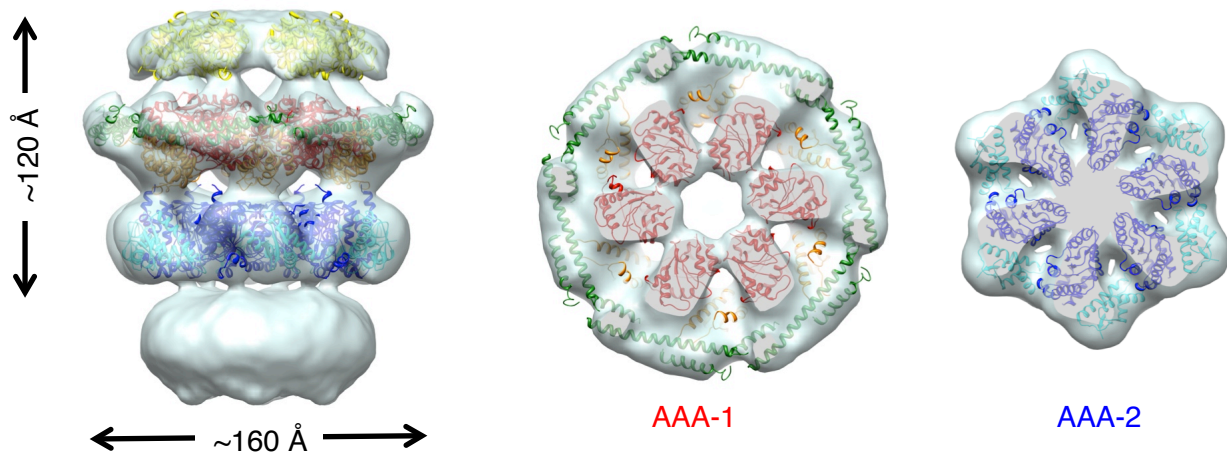
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974 PDB entry code 4CIU

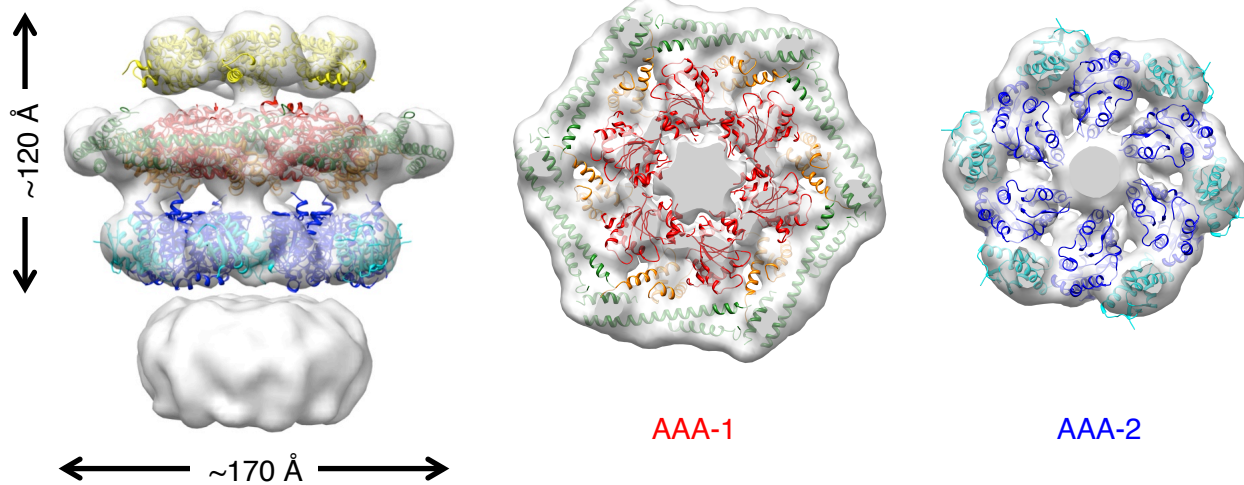
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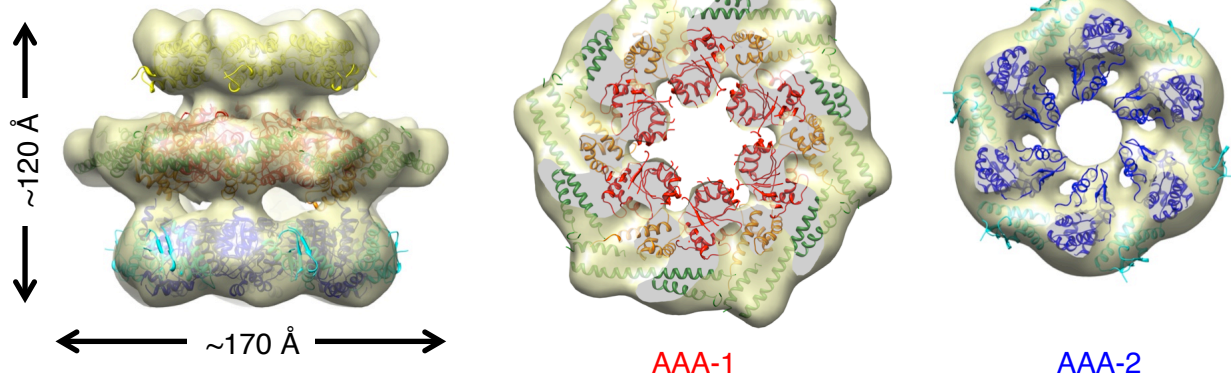
A HAP-ClpP (negative stain)



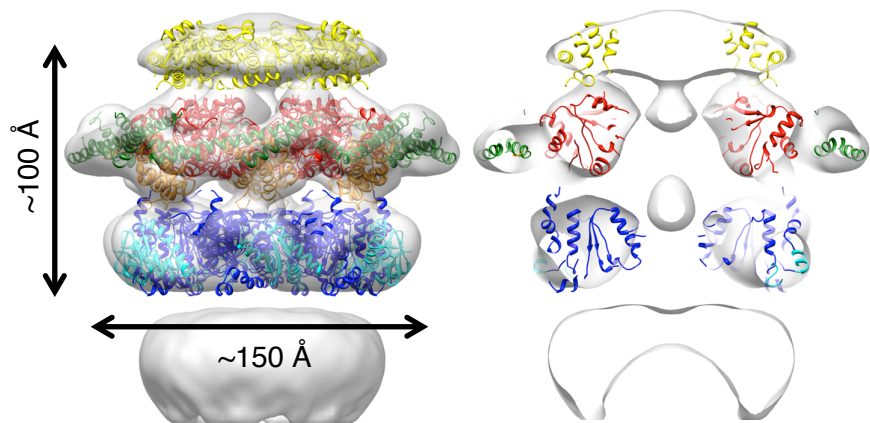
B BAPtrap-ClpP (cryo)



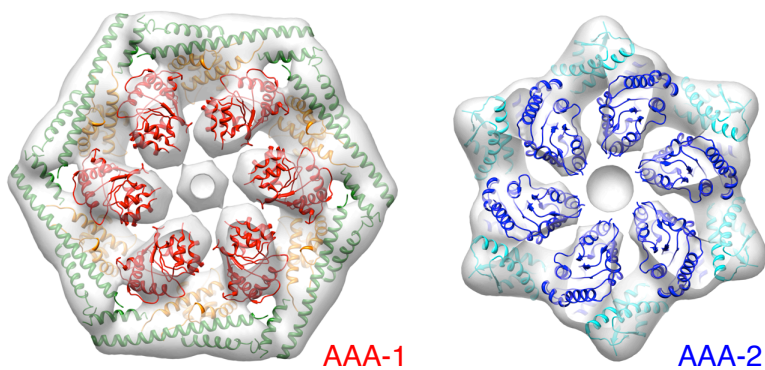
C ClpB (cryo)



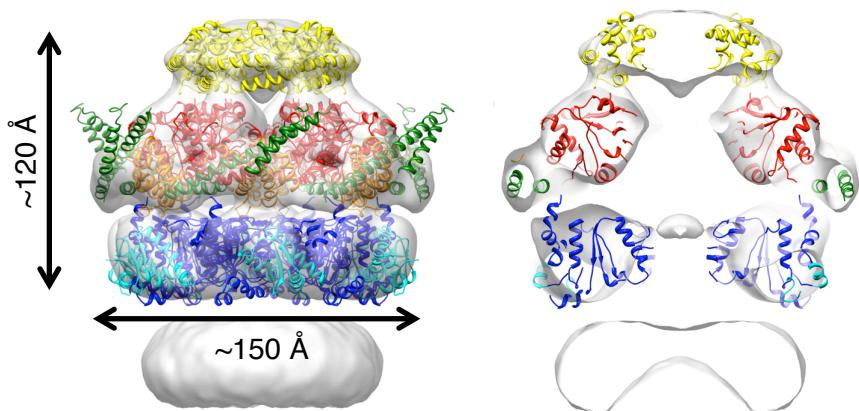
A Repressed E432A



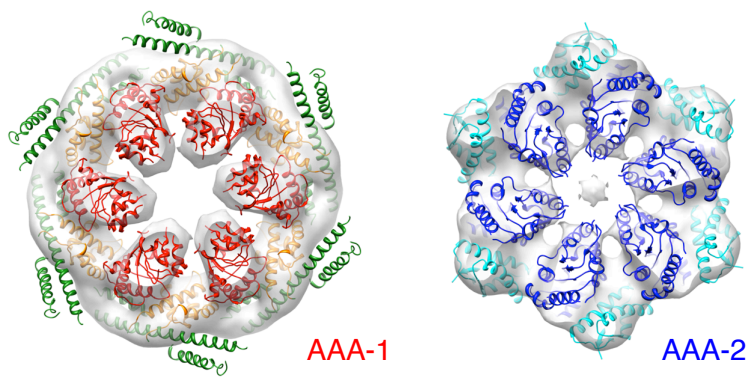
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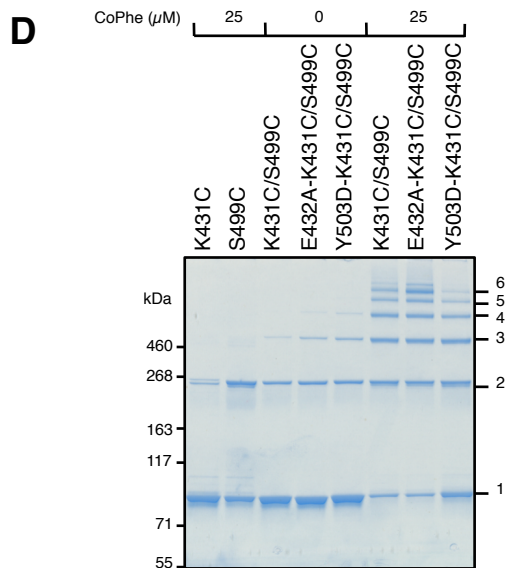
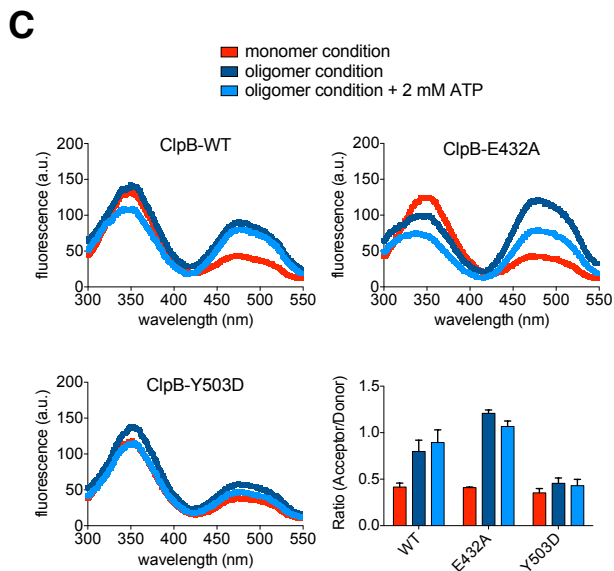
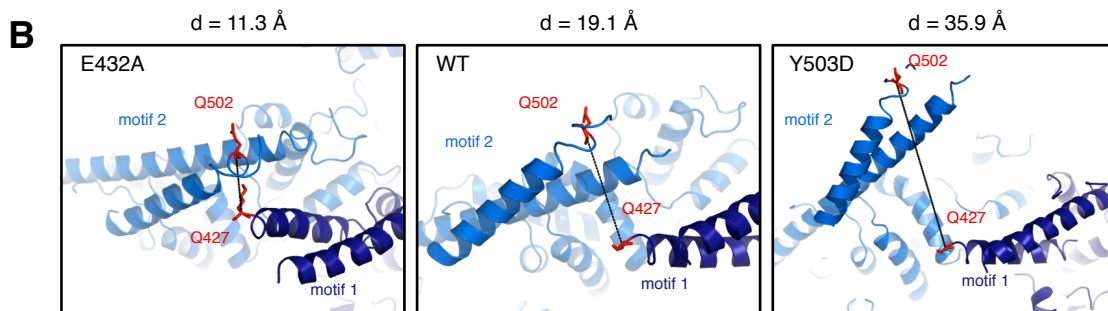
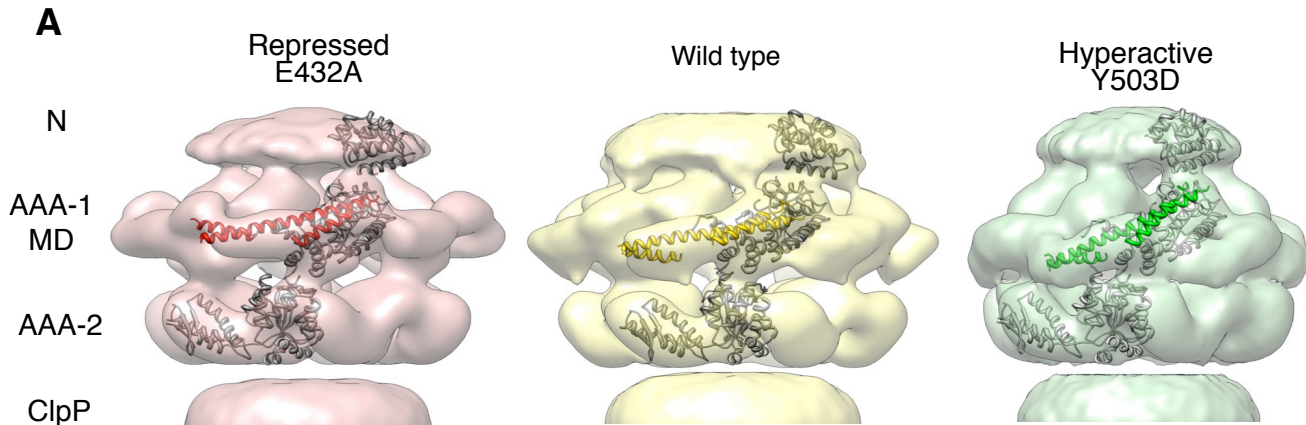


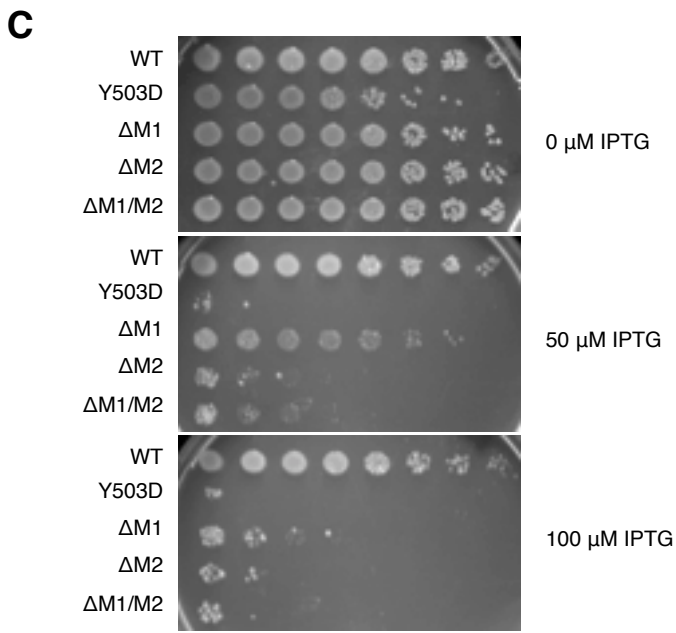
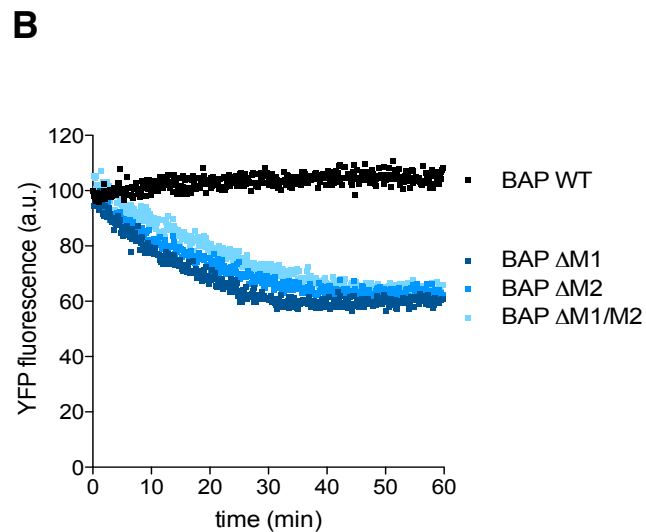
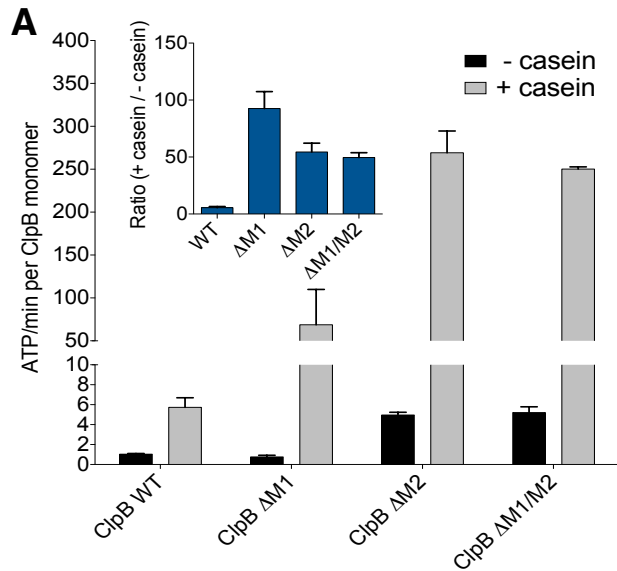
C Hyperactive Y503D

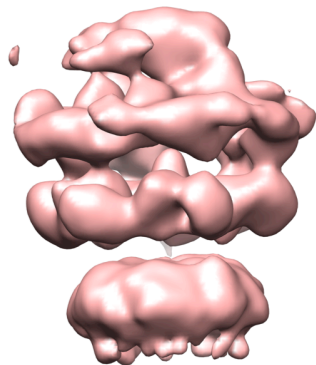


D







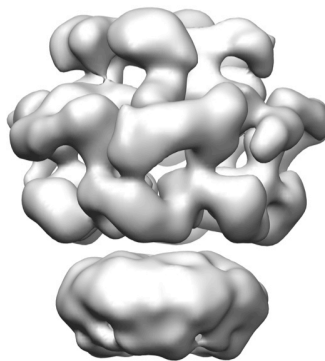
A

Repressed
E432A

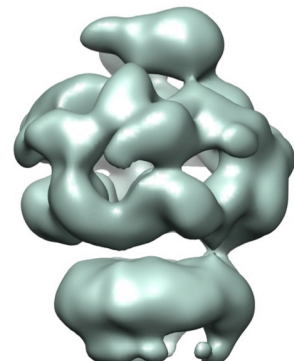
ND
AAA-1/MD

AAA-2

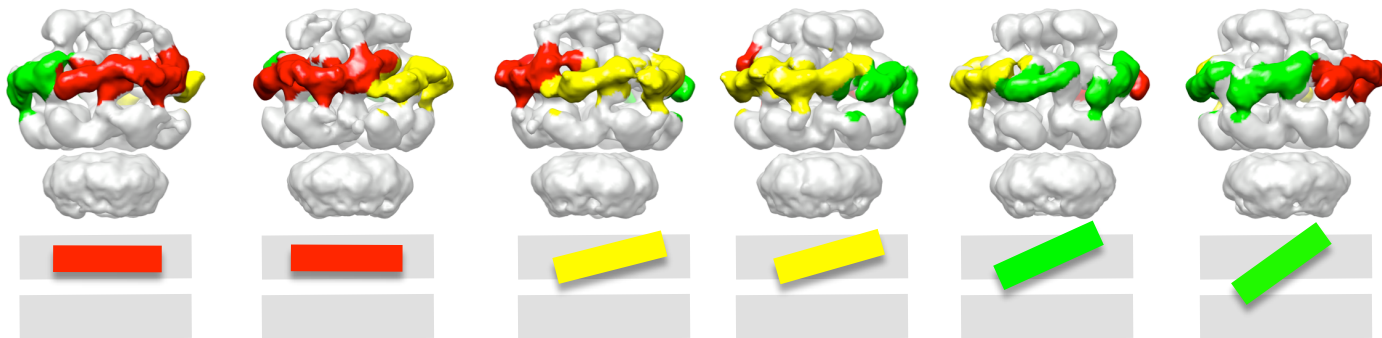
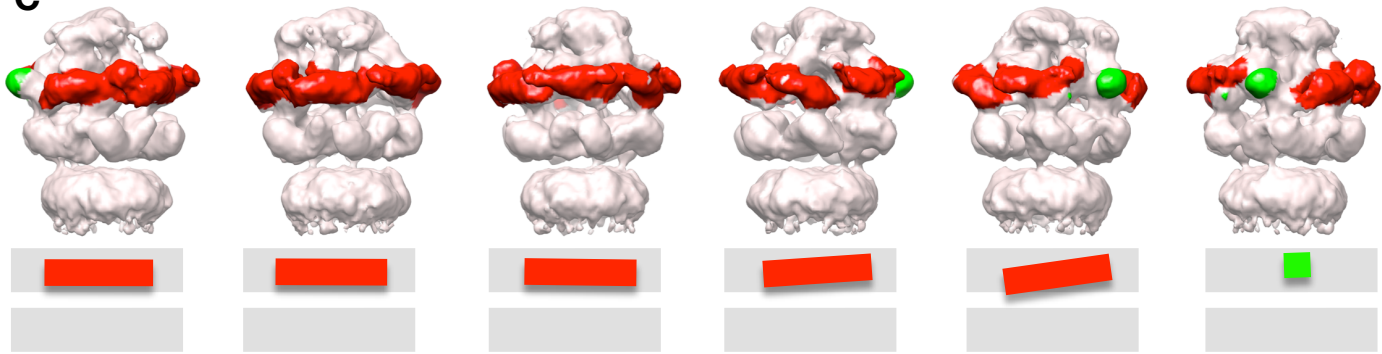
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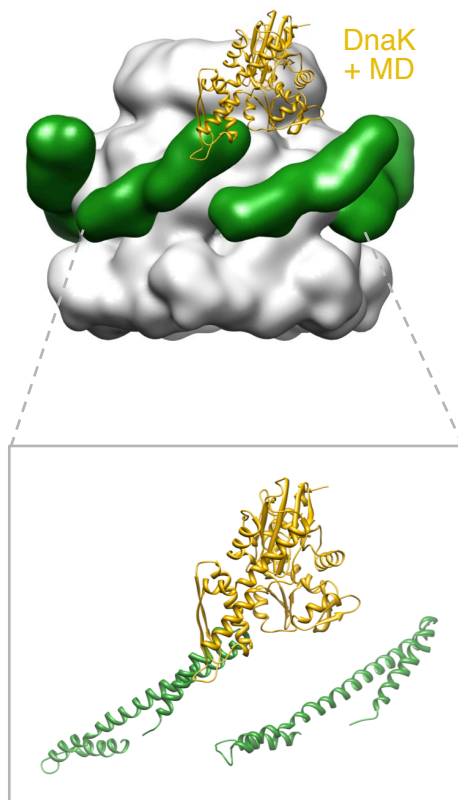
Wild type



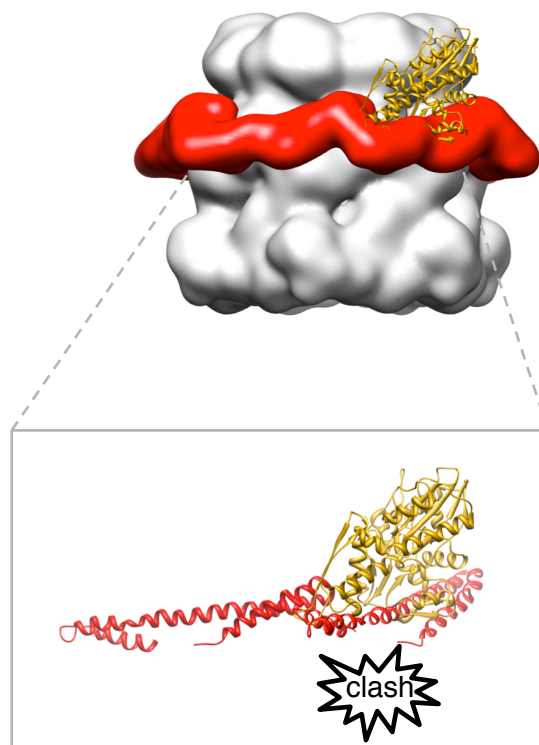
Hyperactive
Y503D

B**C**

A Tilted MD
(hyperactive state)



Horizontal MD
(repressed state)



B

